RESEARCH REPORT No. 1

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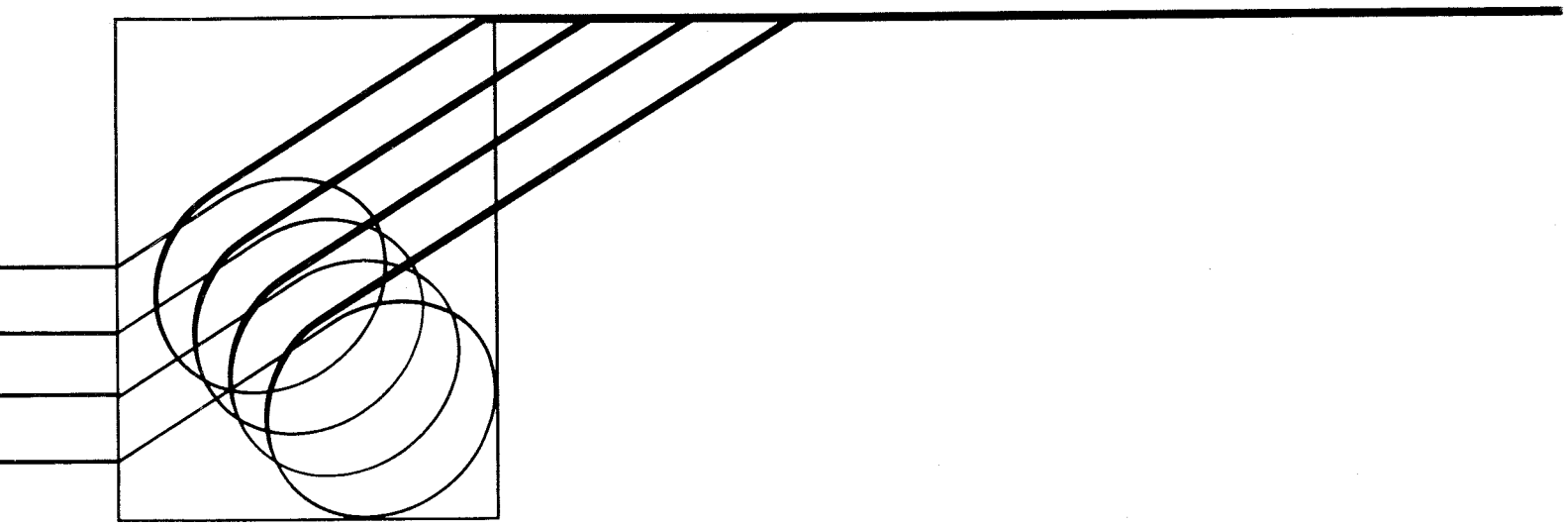

Estimation of Risk of Glucose 6-Phosphate Dehydrogenase Deficient Red Cells to Ozone and Nitrogen Dioxide

Report of the Institute's Health Review Committee

Investigator's Final Report

By Marie A. Amoruso, Ph.D.

Department of Environmental and Community Medicine
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HEALTH EFFECTS INSTITUTE

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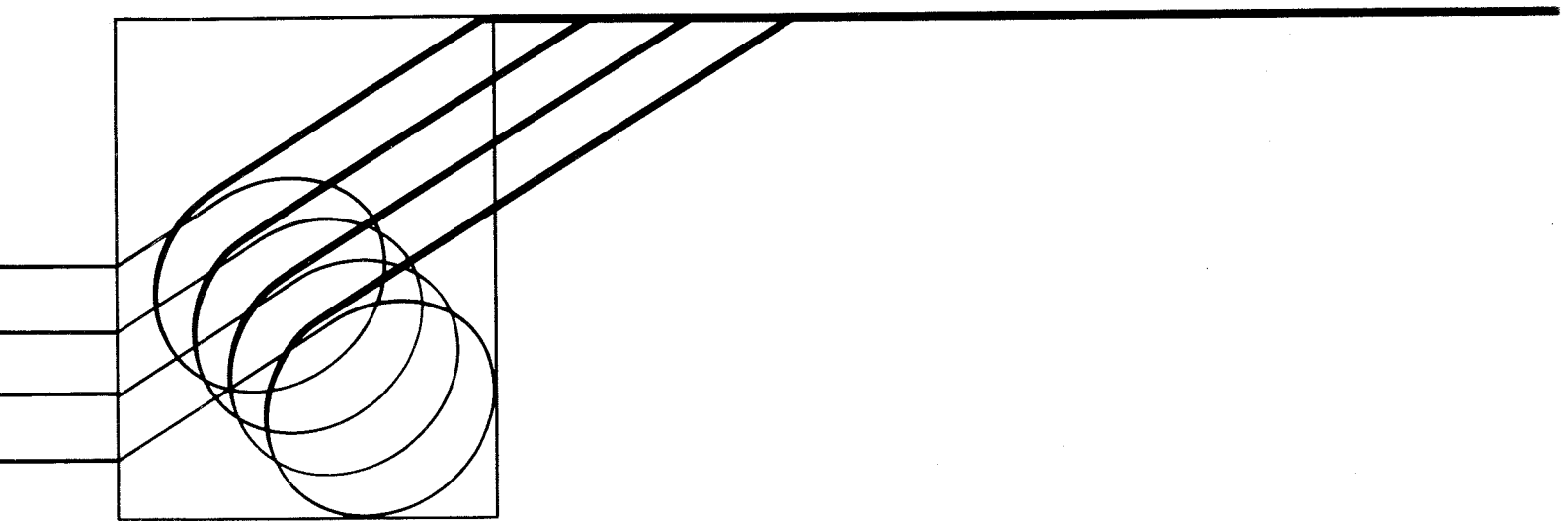

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HEALTH EFFECTS INSTITUTE

The Health Effects Institute (HEI) is a non-profit corporation founded in 1980 to assure that objective, credible, high-quality scientific studies are conducted on the potential human health effects of motor vehicle emissions.

Funded equally by the U.S. Environmental Protection Agency (EPA) and 24 automotive manufacturers or marketers in the United States, HEI is independently governed. Its research projects are selected, conducted, and evaluated according to a careful public process, including a rigorous peer review process to assure both credibility and high scientific standards.

HEI makes no recommendations on regulatory and social policy. Its goal, as stated by former EPA Administrator William D. Ruckelshaus, is "simply to gain acceptance by all parties of the data that may be necessary for future regulations."

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Foreword

A MESSAGE FROM THE BOARD OF DIRECTORS

This document marks the beginning of a new phase in the Health Effects Institute. Five years ago, we were asked by a group of dedicated individuals in the federal government and the automotive industry to help develop a new scientific institution. Its mission was to be straightforward: to become the center for research on the health effects of automotive emissions in the United States. The institution was to be founded on simple principles: it would be independent of its sponsors and committed to the pursuit of truth, even if that truth were painful; it would try to involve the best scientific talent available to work on the most important scientific problems related to potential human health problems from automotive emissions; it would be structured to ensure that its work was credible to often competing factions and to the public at large. The first research was commissioned two years ago, and the results of one experiment and its evaluation are presented herein.

That such an institution was necessary and that both public and private institutions so willingly provided significant resources speak eloquently about the public policy problem being addressed. Science had become enmeshed in the adversary process that seems to be endemic to our public discourse. We believed then, and now, that it was often customary to attack technical aspects of a problem when the real dispute was over the social policy implications. We also recognized that science had all too often become a tool of those whose minds were already made up. The experiment of the Health Effects Institute, then, consists in a determined attempt to remove science from the adversary process, to demonstate that science and policy analysis, while intertwined, have different demands, and to ensure that science is, in fact, an important element in the development of rational regulatory policy. We do not claim that good science guarantees good public policy, but we do believe it is an essential prerequisite.

What do we mean when we invoke the term "good science?" Contrary to the notion that there exists some "silver bullet" study that clarifies all points, we know that the process of science only gradually brings order from chaos. "Good science" is the creative testing of ideas, the first attempts to grope toward new paradigms that explain more of the world around us, as well as the attention to detail and scholarship that are necessary when the objects of our attention are bridges or, perhaps, regulatory standards. In building HEI, we have tried hard not

to focus too narrowly on short-term demands or the pressures of the moment. Rather, we have woven together a community of scholars and investigators who care about the problems of the day, to be sure, but who also care about the fabric of the scientific enterprise. Equal devotion to relevance and to the pursuit of good science is the only sure way to lay the foundations of rational public policy.

Archibald Cox

William O. Baker

Donald Kennedy

June, 1985

Preface

THE HEALTH EFFECTS INSTITUTE AND ITS RESEARCH PROCESS

The Health Effects Institute is a non-profit corporation which, according to its charter, is "organized and operated . . . specifically to conduct or support the conduct of, and to evaluate, research and testing relating to the health effects of emissions from motor vehicles."

It is organized to pursue this purpose in the following ways:

Independence in Governance

HEI is structured to assure credible scientific investigation on the issues it selects. It is governed by a three-member board of directors whose members are William O. Baker, Chairman Emeritus of Bell Laboratories and Chairman of the Board of Rockefeller University, Archibald Cox, Carl M. Loeb University Professor (Emeritus) at Harvard University, and Donald Kennedy, President of Stanford University. Professor Cox chairs the Board. These individuals, who select their own successors, were initially chosen, after consultation with industry and other individuals, by then Environmental Protection Agency Administrator Douglas M. Costle. The current Administration has reiterated its support of the Institution.

Two-Sector Financial Support

The Institute is financed through a unique mechanism. It receives half of its funds from the United States government through the Environmental Protection Agency and half from the automotive industry. Twenty-four leading manufacturers of vehicles or engines that are certified for use on U.S. highways contribute to the Institute's budget in shares which are proportionate to the number of vehicles or engines they sell.

Research Planning and Project Evaluation

HEI is structured to define, select, support and review research to promote the application of the best in scientific research and to better define the possible health effects of mobile source emissions. Its research program is devised and selected by the Health Research Committee, a multi-disciplinary group of scientists who are knowledgeable about the complex problems involved in determining the health effects of mobile source emissions. The Committee seeks advice from its sponsors, and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee has defined an area of inquiry, the Institute announces to the scientific community that requests for application are available. Applications are reviewed first for scientific quality by an appropriate expert panel. They are then reviewed by the Health Research Committee both for quality and for relevance to the mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, which also reviews the procedures and assures the independence and quality of the selection process.

When a study is completed, a draft final report is reviewed by a separate Health Review Committee, which has no role in the selection process. This group assesses the scientific quality of each study and evaluates the contribution of the research to unsolved scientific questions. The study is assigned about a year in advance of completion to a member of the Review Committee, who acts as "primary reviewer." When the report is received, the primary reviewer directs a peer review which involves: (1) the referral of the report to appropriate technical experts and, when appropriate, (2) the involvement of the Review Committee biostatistician who reviews the report to determine whether the data support the conclusions. The primary reviewer then drafts a review which is examined by the full Review Committee, revised as necessary, and made available to the sponsors and to the public along with the final report after evaluation by the Board. All HEI investigators are urged to publish the results of their work in the peer reviewed literature. The timing and nature of HEI Report release is and will be tailored to ensure that the Review Committee's report does not interfere with the journal publication process. The report of the Review Committee will be as thorough as necessary to explain any individual report. Its nature and content may change from study to study.

Part 1. Report of the Health Review Committee

SUMMARY

Acute hemolytic anemia induced by oxidant drugs, such as the antimalarial agent primaquine, is associated with a deficiency in glucose 6-phosphate dehydrogenase (G6PD), an X-linked inheritable characteristic. Such hemolytic response is believed to be a result of depletion of glutathione and other reducing compounds in the red blood cell. Calabrese has hypothesized that G6PD-deficient individuals may be at an increased risk of hemolysis from exposure to low levels of oxidants such as ozone; the hypothesis is based on *in vitro* data on the levels of glutathione in erythrocytes from normal and G6PD-deficient individuals, and information on glutathione levels in human volunteers exposed to ozone. In 1982, Goldstein and Amoruso proposed a study to HEI aimed at experimentally testing the Calabrese hypothesis. The study proposed to link together two sets of *in vivo* data (Alving's data on dose-response of primaquine in G6PD-deficient human volunteers and Miller's data on the rate of delivery of ozone to the lower respiratory tract) by an *in vitro* model (exposure of human G6PD-deficient erythrocytes to primaquine, ozone and nitrogen dioxide). The results obtained by the investigators, as reported by Dr. Amoruso in Part II of this report, demonstrate that: exposure of G6PD-deficient mice to high levels of ozone and nitrogen dioxide had no effect on glutathione levels; exposure of G6PD-deficient human erythrocytes to high levels of ozone and nitrogen dioxide *in vitro* produced only a small (clinically insignificant) decrement in glutathione levels; and, exposure to phenylhydrazine produced a decrease in hematocrit and glutathione levels in normal and G6PD deficient human erythrocytes *in vitro* as well as in mice *in vivo*. The investigators conclude that the likelihood that ambient levels of ozone and nitrogen dioxide produce significant oxidant stress in G6PD-deficient individuals is very low. However, the investigators did not fully use the Miller data — and the substitution of phenylhydrazine in the place of primaquine precluded the use of the Alving data. Therefore, the original intention of the investigators to construct a logically organized extrapolation matrix was not fulfilled. Several other limitations also bear on the interpretation of the study. Nevertheless, the study supports the contention that ambient levels of ozone and nitrogen dioxide do not pose a significant risk of hemolysis to G6PD-deficient individuals.

INTRODUCTION

In the summer of 1982, the Health Effects Institute (HEI) issued a Request for Applications (RFA 82-3) soliciting studies on "models of susceptible populations." (1) In the fall of 1982, Dr. Bernard Goldstein and Dr. Marie Amoruso of the Department of Environmental and Community Medicine, University of Medicine and Dentistry of New Jersey, Rutgers Medical School, proposed a project, entitled "Estimation of Risk of Glucose 6-phosphate Dehydrogenase Deficient Red Cells to Ozone and Nitrogen Dioxide." (2) HEI approved the project, which began in the spring of 1983. In August of that year,

Dr. Goldstein left the project, after his appointment as Assistant Administrator for Research and Development of the United States Environmental Protection Agency. At that time, with HEI's concurrence, Dr. Amoruso assumed full responsibility for the project as principal investigator (3). The contract for the study authorized $52,000 for a one-year period. Dr. Amoruso completed the project in the fall of 1984. After review by HEI, she submitted her final report to HEI on March 14, 1985 (4). This paper is a review of the study and of the final report, as an aid to the sponsors of HEI and the public (5).

This paper proceeds as follows. The next section, the third in this paper, discusses the statutory provisions and legislative history of the relevant sections of the Clean Air Act (6). The fourth section discusses the scientific context of this project, as well as the goals of the investigators in proposing and the objectives of HEI in funding this work. The fifth section contains the major technical evaluation of the work. It discusses the relationship between the original proposal and the work performed by the investigators, the contribution of this project to the evaluation of the relationship between G6PD deficiency and hemolysis† from exposure to nitrogen dioxide and ozone as well as criticism of the project's approach and implementation. The sixth section briefly addresses the uncertainties that remain after this project, and suggests additional research that might be conducted by HEI or other institutions to advance the state of knowledge in this area. Finally, the last section addresses the value this work might have in assessing risk.

THE CLEAN AIR ACT AND SUSCEPTIBLE POPULATIONS

Section 109 of the Clean Air Act specifies, in part, that "national primary ambient air quality standards...shall be ambient air quality standards the attainment and maintenance of which in the judgment of the Administrator of EPA, based on such criteria and allowing an adequate margin of safety, are requisite to protect the public health." (7) The legislative history of the Act makes it clear that the Environmental Protection Agency is required to consider the health of sensitive subgroups of the population in setting these ambient air quality standards. A report on the legislation by the Senate Committee on Public Works states, "An ambient air quality standard, therefore, should be the maximum permissible ambient air level of an air pollution agent or class of such agents (related to a period of time) which will protect the health of any group of the population." (8) The definition of such groups is not entirely clear, although the report does specify that "included among those persons whose health should be protected by the ambient standard are particularly sensitive citizens such as bronchial asthmatics and emphysematics who in the normal course of daily activity are exposed to the ambient environment." (9)

† Technical terms marked with a dagger are explained in the glossary, page 41.

Further, the report states that "in establishing an ambient standard necessary to protect the health of these persons, reference should be made to a representative sample of persons comprising the sensitive group rather than to a single person in such a group." (10)

There is, as yet, no definitive way to determine, *a priori*, all of the sensitive subgroups in the population. However, on general principles, the human population is expected to include a number of subgroups more vulnerable to the effects of mobile source emissions than "normal" individuals. Such vulnerability may result from variations in genetic, physiological, dietary, behavioral or other factors. In 1978, Calabrese (11) attempted to estimate the size of human populations at special risk from the effects of environmental pollutants. He hypothesized twenty-eight genetic conditions that may predispose human populations to environmental risks, and discussed the potential importance of developmental, dietary and behavioral factors.

G6PD DEFICIENCY AND OBJECTIVES OF THE HEI PROJECT

The Problem of G6PD Deficiency

Glucose 6-phosphate Dehydrogenase (G6PD) is an enzyme that is important for the metabolism of glucose and other sugars. It is also involved in the metabolism of glutathione[†], a substance that is important for detoxifying a number of drugs and other chemicals. G6PD first came to the attention of the medical community when several black patients receiving therapeutic doses of antimalarial drugs of the 8-aminoquinoline family (such as primaquine and pamaquine) suffered acute hemolytic anemia[†] (12). This effect was not observed in the general population using the drug therapeutically. The hemolytic response to pamaquine and other 8-aminoquinoline drugs is a potentially life-threatening condition.

The sensitivity to primaquine was subsequently shown to be associated with a group of enzyme deficiencies, the most common of which results in a decrease in G6PD activity (13). It has been estimated that about 2 million US black males (approximately 14%) are affected by G6PD deficiency. Further research has shown G6PD deficiency to be present in other racial groups as well (14). In addition to the 8-aminoquinoline antimalarials, G6PD-deficient individuals are also sensitive to a number of other drugs and chemicals (see Table A.1), although only a small fraction of the exposed subjects exhibit the life-threatening symptoms of acute hemolytic anemia.

There are a number of variants of G6PD deficiency; the A^- variant is the most important for this report because it is the one most common in the US population. The G6PD deficiencies are transmitted as an

X-linked† characteristic and therefore are more prevalent among males. Each variant of G6PD deficiency results in a different but characteristic level of enzyme activity in erythrocytes† (15). Normal individuals exhibit no hemolysis after administration of pamaquine, although four times the usual dose of a similar drug, primaquine, can cause some hemolysis in individuals who have normal G6PD activity. The mechanism by which G6PD deficiency causes hemolysis is still not completely understood. (See Discussion in Appendix.)

G6PD Deficiency and Air Pollution: The "Calabrese Hypothesis"

Little is actually known about the effects of common air pollutants, such as ozone and nitrogen dioxide and its oxidant products, on individuals with G6PD deficiency. The proposed mechanisms of action of air pollutants on humans are believed to be indirect. As a highly reactive chemical, ozone is not expected to exist for any appreciable time after contact with lung tissue, and is not expected to penetrate the pulmonary capillary-alveolar membrane barrier to reach the blood. However, *in vivo*† exposure to ozone (0.5 ppm for 2.75 hours) has been reported to lower the glutathione level of erythrocytes in normal individuals by 14 percent (16). The mechanism for the effect of ozone on red blood cells *in vivo* is poorly understood. It has been suggested that lipid-peroxidation may play a role in ozone-mediated effects.

In 1977, E.J. Calabrese first hypothesized that individuals with G6PD deficiency are hypersusceptible to hemolysis from exposure to ozone (17). Although this hypothesis has been expanded to include other environmental pollutants with oxidizing potential, such as chloramines (18), the hypothesis is based on extrapolation of data from laboratory experiments with ozone and certain drugs.

Calabrese developed his hypothesis from observations made by other investigators *in vivo* in normal and G6PD-deficient individuals and from *in vitro*† experiments with human erythrocytes. A brief description of these experiments, inferences and conclusions follows:

Data from Normal Individuals: After inhalation of ozone, normal individuals have decreased levels of glutathione (a 14 percent reduction after 2.75 hours of exposure to ozone at 0.5 ppm) (16), and exhibit increased osmotic fragility† of their erythrocytes. Red blood cells from normal individuals adapt to ozone exposure by increasing the activity of the hexose monophosphate shunt† including the G6PD activity. Calabrese viewed these effects as reflecting a "homeostatic regulatory compensatory" response that would not be possible for G6PD-deficient individuals.

Data from G6PD-Deficient Individuals: Erythrocytes of G6PD-deficient individuals have a decreased capacity to produce $NADPH^{+}$ by the hexose monophosphate shunt. Since NADPH is necessary for the

reduction of glutathione, they have a decreased ability to reduce glutathione. In the absence of stressors, erythrocytes of the A^- variant of G6PD-deficient individuals have only 60-70 percent of the glutathione of normal individuals (12, 19). During the acute hemolytic stage of a G6PD-deficient person's reaction to primaquine, these already lowered glutathione levels can be reduced a further 14-20 percent (19, 20).

Data from In Vitro Experiments: In *in vitro* glutathione stability test, when normal erythrocytes are incubated with the oxidant drug, acetylphenylhydrazine (APH), glutathione levels often drop by approximately 20 percent. Under these same conditions, glutathione levels in erythrocytes from G6PD-deficient individuals often drop 80 percent and may approach 100 percent depletion (18, 19).

Calabrese reasoned that these data suggest glutathione levels in G6PD-deficient individuals may be reduced by 56 percent upon exposure to ozone. Although the calculations are not presented in his papers, it can be inferred that Calabrese assumed that the *in vitro* comparison of normal and G6PD-deficient erythrocytes challenged with APH would yield effects proportional to those that would be observed *in vivo* upon ozone exposure. That is:

(% reduction GSH_{normal}) / (% reduction GSH_{G6PD}) *in vitro* (APH)

= (% reduction GSH_{normal}) / (% reduction GSH_{G6PD}) *in vivo* (O3)

or

$$20\% / 80\% = 14\% / x$$

$$x = 56\%$$

Accordingly, Calabrese *predicted* that a G6PD-deficient individual with a typical glutathione (GSH) of 40 mg% and an ozone exposure similar to that of normal people, as reported by Buckley *et al.* (1975), would have a GSH level decreased by 56 percent to 18 mg% (16, 18). Because values of glutathione below 20 mg% are usually associated with hemolysis, Calabrese contended that G6PD-deficient individuals exposed to ozone at levels above 0.5 ppm are at risk for hemolysis. The current ozone standard is 0.12 ppm/1 hour averaging time, although significant parts of the United States are not in compliance and so may have ambient ozone levels substantially exceeding 0.12 ppm.

A Summary of the Problem

- Almost two million black American men have G6PD deficiency (A^- variant).

- When exposed to significant oxidant stress, humans with this deficiency exhibit significant hemolysis, which can result in substantial health effects, including acute hemolytic anemia, a potentially life-threatening condition.

- According to a hypothesis put forth by E.J. Calabrese, exposure to levels of ozone in the ambient environment could contribute to such health effects.

- If this hypothesis were true, it would have significant implications for regulation under the Clean Air Act, because the Act requires protection, with an adequate margin of safety, of the health of susceptible populations.

Objective of the Study Proposed by Goldstein-Amoruso

In their September, 1982, proposal to HEI, Goldstein and Amoruso challenged the Calabrese hypothesis:

> "Based on inferences drawn from our own previous studies evaluating the effects of inhaled ozone on the red cells of animals and men (21, 22, 23, 24, 25, 26, 27) and our interpretation of the relevant data concerning the pathogenesis† and health implications of the hematological pathogenesis and health implications of the hematological effects of G6PD deficiency, we believe that the risk that ambient ozone exposure will have a measurable effect on hematological parameters of black Americans with G6PD deficiency is negligible." (28)

The proposal itself outlined both the major objections to the hypothesis, and the objectives of the investigators:

> "In order for G6PD deficiency to put at risk a circulating blood cell for an adverse effect of ozone, either ozone, or an oxidation product of ozone must penetrate into the red blood cell. That a highly reactive compound such as ozone could traverse the mucous-aqueous lining of the lower bronchial tree, where it is preferentially deposited, go through the alveolar and the capillary lining, enter the blood stream and penetrate into the red cell seems unlikely. It is, however, conceivable that an ozone-induced product might arrive at the circulating red cell and that such a product could have oxidizing potential to which G6PD-deficient red cells would be particularly susceptible. In fact, studies in this laboratory demonstrated that a catalase-reacting material, presumably hydrogen peroxide, could be detected in the blood of rats or mice inhaling ozone (29). However, the dose of ozone required for this observationwas very high, being 5 ppm ozone or greater. This

level produces significant pulmonary edema and is well above the U.S. primary air quality standard of 0.12 ppm for one hour. It is of course conceivable that a different assay might detect an oxidant stress at lower ozone levels. Menzel *et al.* have noted Heinz body formation, consistent with an oxidant effect, in the blood of ozone exposed mice which they ascribe to circulating fatty acid ozonides (30). However, we have been unable to replicate this finding (unpublished data). Our studies of red cell effects in man and animals exposed to ozone have led to the hypothesis that such effects occur primarily at the cell membrane and that lung lipid peroxide decomposition products, particularly carbonyl derivatives, are perhaps responsible for the finding (21, 22, 23, 25). Such carbonyl compounds are not oxidizing agents in the usual sense and it is unlikely that G6PD-deficient red cells would be more susceptible. Our findings include a decrease in activity of acetylcholinesterase, a red cell membrane enzyme whose active site is oriented to the outside of the membrane (22) and which is particularly susceptible to free radicals (31). A 50 percent decrease in mouse red cell acetylcholinesterase following inhalation of 8 ppm ozone for four hours was not associated with a statistically significant decrease in red cell GSH, despite the fact that this dose approaches the LD_{50} (22). However, this does not rule out the possibility that a decrease in GSH would occur in G6PD-deficient red cells at lesser ozone levels. Other red cell membrane effects in our laboratory include decreased agglutinability of rat red blood cells by concanavalin A following inhalation of 0.5 ppm ozone for two hours (26). In humans exposed to 0.5 ppm ozone for two hours the results were equivocal (27).

"Studies by Buckley *et al.* (16) in humans experimentally exposed to ozone also demonstrated a decrease in red cell acetylcholinesterase activity (16). As pointed out by Calabrese (32), Buckley *et al.* also observed a 14 percent decrease in red cell GSH and a 20 percent increase in G6PD activity consistent with an oxidant effect and a compensatory G6PD response. *As Buckley* et al. *did not observe a reticulocytosis, their report of an increased red cell G6PD activity following ozone exposure in man remains unexplained...Dr. Calabrese has used Dr. Buckley's data as a basis to project a substantial risk of adverse hematological effects in G6PD-deficient individuals exposed to ambient levels of ozone* (emphasis added). However, our own calculations based on the available data suggest that it will require two orders of

magnitude above ambient ozone levels to produce hematological affects in these individuals. Rather than argue the point, we feel it more important to obtain data useful to refine the extrapolations." (33)

The major aim of the proposal, then, was straightforward:

> "To determine the maximum extent ozone is likely to affect the hematological status of individuals with glucose 6-phosphate dehydrogenase (G6PD) deficient (A^- variant) red blood cells." (34)

In sponsoring this work, HEI explicitly shared these objectives. HEI did, however, add to the experimental design (using the same protocol) an evaluation of the effects of nitrogen dioxide. Because nitrogen dioxide also has potential oxidant effects, HEI believed that its addition to these sets of experiments would further enhance the value of this study.

The Proposed Protocol

In order to estimate the potential effect of ozone and nitrogen dioxide on G6PD-deficient human red cells *in vivo*, Goldstein and Amoruso proposed to link together two data sets by an *in vitro* model (Table 1.1). The data sets are the studies by Alving *et al.* in which the hematological effects of known doses of primaquine were carefully studied in G6PD-deficient human volunteers (15), and the work by Miller *et al.* which estimated the rate of delivery of ozone to the lower respiratory tract in man (35). The proposed *in vitro* study involved exposure of human G6PD-deficient erythrocytes *in vitro* to primaquine or to ozone. By comparing the level of ozone with that of primaquine needed to produce a hematologic response *in vitro*, extrapolating the primaquine dose to that reported by Alving as causing an effect, and relating the ozone dose to external exposure by means of the Miller data, the investigators proposed to estimate the level of inhaled ozone required to produce *in vivo* hemolysis in humans. In a parallel approach, the investigators proposed to compare the doses of ozone and primaquine required to produce hemolysis *in vivo*, and red cell effects *in vitro*, in mice.

In funding this study, HEI recognized that extrapolation uncertainties would be present. There was a clear sense, however, that if, as the investigators expected, the amount of ozone delivered to the erythrocytes is orders of magnitude lower than the ambient ozone levels, then the uncertainties would not be significant. If, contrary to the investigators' expectation, the study demonstrated that the amounts of ozone necessary for hemolysis were close to ambient levels, further studies would be necessary. The focused nature of the inquiry, the

reputations of the investigators, and the limited financial cost of the study made the proposal attractive.

TECHNICAL EVALUATION

Conclusions of the Study

Dr. Amoruso's report to HEI is contained in full in Part 2 of this publication. The study design required the completion of a dose-response matrix *in vivo* and *in vitro* in mice and in humans for primaquine, ozone and nitrogen dioxide. Table 1.1 summarizes the proposed experiments. The report, as received by HEI, includes *in vitro* studies in human erythrocytes using ozone and nitrogen dioxide, but with phenylhydrazine rather than primaquine. In addition, the report describes *in vivo* studies in mice exposed to ozone and nitrogen dioxide, and phenylhydrazine given intraperitoneally[†] rather than primaquine by mouth. Table 1.2 summarizes the studies performed by the investigator. The tabulated data in Dr. Amoruso's report show no change in glutathione levels in mice exposed to high levels of ozone and nitrogen dioxide. The investigators report that in human erythrocytes from G6PD-deficient individuals exposed to high levels of ozone and nitrogen dioxide *in vitro*, there was a drop in glutathione levels; however, the drop was too small to be considered clinically significant. The investigators also report that phenylhydrazine produced a dose-dependent depression in hematocrit and glutathione *in vivo* in mice, and *in vitro* in human erythrocytes.

On the basis of these investigations, Dr. Amoruso concludes that the absence of *in vivo* effects in mice exposed to high levels of ozone and nitrogen dioxide and the observed effects of phenylhydrazine *in vivo* in mice and *in vitro* in human erythrocytes, indicate that the likelihood that ambient levels of ozone and nitrogen dioxide produce significant oxidant stress in G6PD individuals is very low. While the study tends to support the contention that Calabrese's hypothesis in incorrect, there are insufficient data in the report to complete the extrapolation to man.

Table 1.1
Experiments Proposed

	Mouse		Man	
	In Vitro	In Vivo	In Vitro	In Vivo
PMQ	P	P	P	1
Ozone	P	P	P	2
Nitrogen Dioxide	3	3	3	3

Table 1.2
Experiments Performed

	Mouse		Man	
	In Vitro	In Vivo	In Vitro	In Vivo
PMQ	N	N	N	N
PHZ	N	Y	Y	4
Ozone	N	Y	Y	5
Nitrogen Dioxide	N	Y	Y	6

P: Proposed.
N: Experiment not reported.
Y: Experiment reported.
1: Data available from Alving *et al.* (15).
2: Data available from Buckley *et al.* in normal subject, and from Miller *et al.* (35) on delivery of ozone in the human lung.
3: HEI added this experiment to the investigation.
4: No discussion in the final report.
5: Extrapolation could not be completed; see text for discussion.
6: No discussion in the final report.

The Relationship of the Study to the Original Design

The original plan for the study contemplated a series of experiments linked by an overall matrix (Table 1.1). In several respects, the actual execution of the study did not complete the proposed protocol (Table 1.2).

Because of the difficulty of obtaining sufficient blood from G6PD-deficient mice, the investigators did not perform *in vitro* studies in mice. Furthermore, phenylhydrazine was substituted for primaquine (rather than being done in parallel), thus making it impossible to utilize the data of Alving *et al.* in humans exposed to primaquine. In addition, information on dose-response of phenylhydrazine in humans is not discussed. The dose of phenylhydrazine administered to G6PD-deficient mice was three times greater than a dose that has been shown to cause acute hemolysis in clinical studies reported by Kellermeyer *et al.* (36) in a G6PD-deficient male population. An additional difficulty encountered by the investigators, which might have been anticipated, is that primaquine needs to be metabolically activated to exhibit toxic effects; thus *in vitro* experiments with primaquine are extremely difficult and were not reported by the investigator. The study made only limited use of data of Miller, as proposed, to estimate the dose of ozone to the lower respiratory tract *in vivo*, although the final report does contain a calculation of the ozone dose to the blood in man.

In addition, the human *in vitro* studies use an exposure of G6PD-deficient red blood cells to ozone; however, the experimental design does not permit an estimate of the amount of ozone reacting with the red blood cells, but rather only of the amount removed by the red cells and the medium within the reaction vessel collectively. Therefore, it is impossible to know how much ozone actually contacted and reacted with red blood cells.

Interpretation of the Study

Several critical factors bear on the interpretation of the data:

- Hemolysis is the end result of the effects of ozone (or products generated by ozone) on the red cell membrane. Most of the ozone effects on red cells reported in the literature appear to be due to *membrane* oxidation and not glutathione oxidation. Glutathione oxidation, as reported in the Amoruso study, is not a direct measure of membrane effects.

- The *in vivo* model used mice that have only a relative deficiency of G6PD not nearly as severe as the human A^- variant. (Both the investigator and HEI recognized this fact as a limitation prior to commencement of the studies.) Therefore, the mouse red blood cells might tolerate considerably more oxidant than the deficient human red blood cells. The red blood cell effects of phenylhydrazine are not adequate for assessment of this concern because of both the nature of the exposure (intraperitoneal injection) and the nature of the agent.

- Substantial uncertainty exists in identifying the dose to the target tissue, i.e. the red blood cell, both *in vitro* and *in vivo*, as well as in differentiating anti-oxidant defenses *in vivo* and *in vitro*.

- The Miller data on the penetration of ozone to the lower respiratory tract were used to a very limited extent. In fact, the modelling of ozone and nitrogen dioxide exposure by Miller and co-workers has only recently taken into account the blood pool as a tissue compartment in the lung exposed to ozone. Thus, the appropriate calculations of the amount of ozone reaching the blood pool would be more complex but would probably result in levels lower than that suggested in the report.

- The administration of phenylhydrazine precluded the linking of the data generated in this study to Alving's primaquine data in humans, as originally proposed. Primaquine has a delayed effect because it depends on metabolism for its oxidant effects. Phenylhydrazine is a direct oxidant, and perhaps more comparable to ozone *in vitro* than is primaquine.

- The investigator assumes that there is a stoichiometric† relationship between ozone and glutathione levels, i.e., that ozone has a direct effect on glutathione levels. However, it is questionable whether a stoichiometric relationship should be assumed to apply between ozone and glutathione; ozone may generate free radicals which could initiate a chain reaction and greatly increase the number of molecules that might penetrate to the red blood cells. This point was not considered in the final report.

- The animal exposures were acute and not chronic, but human environmental exposures are usually chronic and not acute. Data on the chronic effects of ozone and nitrogen dioxide would have been useful from a public health perspective. Because a chronic study was not a part of this proposal, this comment is not a criticism of the study but a notation of a limitation of its usefulness.

Despite these considerations, the study adds useful data on an *in vivo* G6PD-deficient mouse model exposed to high levels of ozone and nitrogen dioxide, and it presents worthwhile observations on the *in vitro* effects of ozone and nitrogen dioxide on normal human and G6PD-deficient red blood cells. Although the original intention of the investigators, the construction of a logically organized extrapolation model, was not fulfilled, the study supports the proposition that exposure of G6PD-deficient individuals to ambient levels of ozone and nitrogen dioxide would be unlikely to cause a significant risk in man.

REMAINING UNCERTAINTIES AND ADDITIONAL RESEARCH DIRECTIONS

The Investigators' arguments in the original application were cogent, and the study suggests some additional support for those arguments. It is clear from the foregoing, however, that this research did not completely resolve the issue of G6PD deficiency and hypersusceptibility to ambient levels of ozone and nitrogen dioxide. We are, therefore, left with three alternatives for future consideration:

- Test the question directly with a carefully conducted study of ambient ozone and nitrogen dioxide exposure in G6PD-deficient humans. This would be the most direct test and, considering the outcome of the mouse experiments as reported in this study, would be of acceptable risk. If the observation of Buckley *et al.* (1975) cannot be repeated, there is no basis for the Calabrese hypothesis (16, 17, 18).

- On the basis of the mouse data, accept the unlikelihood that ozone and nitrogen dioxide at ambient levels pose a special risk to persons with G6PD-deficient red blood cells.

- Complete the proposed experimental design by obtaining *in vitro* mouse data and by using the data of Alving and Miller. However, given the problems discussed above with the extrapolation of *in vitro* data, these experiments are not likely to resolve the issue of G6PD deficiency and sensitivity to oxidant stress.

IMPLICATIONS FOR PUBLIC POLICY AND RISK ASSESSMENT

The history of interpretation of the Clean Air Act makes it clear that no single study is often dispositive, but that the cumulative weight of evidence is important (37). This study is suggestive, but not conclusive. It should be possible to undertake a well-conducted human study that could resolve the issue of G6PD deficiency and hypersusceptibility to ozone and nitrogen dioxide exposure at ambient levels.

The question of G6PD deficiency and hypersusceptibility to oxidant stress is not fully resolved by this study. The principal investigators are to be commended, however, for the idea presented in their original proposal, which is an attempt to relate ozone or nitrogen dioxide effects on a quantitative basis in animals and humans. This attempt, although not completed in the present study, is a new direction in ozone and nitrogen dioxide toxicity research.

Appendix

G6PD DEFICIENCY AND HEMOLYSIS

The course of drug-induced hemolysis can be divided into three phases (13, 15): acute hemolysis, recovery, and resistance to further hemolysis during continued treatment. Upon cessation of the treatment, however, the resistance is slowly lost, so that subsequent treatment with the drug will again result in acute hemolysis. This transient resistance to primaquine appears to result from the lower sensitivity of young erythrocytes to the hemolytic effects of primaquine (19). The older erythrocytes are more sensitive to the effect of the drug and are destroyed during the acute hemolysis phase. A concomitant increase in production of erythrocytes is observed.

The younger, more resistant cells provide the basis for the clinically observed resistance to the drug. Because mature, G6PD-deficient erythrocytes are more sensitive to hemolysis, erythrocytes have a shorter average half-life for the duration of drug exposure. As long as administration of the drug continues, with concomitant destruction of the older, more sensitive cells, clinical symptoms of anemia are not observed. When exposure ceases, the older population of cells returns, and the G6PD-deficient individual will again exhibit an acute hemolytic crisis upon readministration of the drug. It should be noted that erythrocytes of G6PD-deficient individuals were found to have shorter half-lives than those of normal individuals, even when the erythrocytes were not subject to known oxidant stressors (19).

The mechanisms of hemolysis in G6PD-deficient individuals under conditions of oxidant stress are not well understood. In addition to the agents discussed in this report, other agents have been reported to induce hemolysis in G6PD-deficient individuals (Table A.1). That the enzyme deficiency was first observed in erythrocytes might have been a result of the importance of G6PD for normal erythrocyte metabolism. G6PD catalyzes the second step of the hexose monophosphate shunt, i.e., the conversion of glucose 6-phosphate to 6-phosphogluconolactone. This conversion requires NADP, which is concomitantly reduced to NADPH, and is the sole mechanism within the mature erythrocyte for the production of NADPH.

Metabolic products of drugs such as primaquine oxidize various molecules in the erythrocytes; in other words, they increase transfer of protons from NADPH, sulfhydryl groups of glutathione and proteins such as hemoglobin, and other cellular constituents. NADPH is required for the reduction of the oxidized form of glutathione and thus for the maintenance of adequate levels of reduced glutathione. Reduced glutathione has been reported to protect hemoglobin and other constituents of erythrocytes from destruction by a variety of oxidant drugs (15). Normal erythrocytes are protected from the adverse effects of oxidant drugs because they are capable of generating sufficient amounts of NADPH by metabolizing glucose by the hexose

Table A.1
Agents reported to be capable of inducing hemolytic anemia in subjects with genetically determined idiosyncratic susceptibility. From Goldstein *et al.* (40).

Primaquine	Quinine
Pamaquine	Quinidine
Pentaquine	p-Aminosalicylic acid
Quinocide	Antipyrine
Sulfanilamide	Acetanilid
Sulfapyridine	Probenecid
Sulfisoxazole	Phenylhydrazine
Sulfacetamide	Acetophenetidin
Sulfamethoxpyridazine	Pyramidone
Salicylazosulfapyridine	Chloroquine
Sulfones (sulfoxone)	Chloramphenicol
Naphthalene	Fava bean
Methylene blue	Viral respiratory infections
Vitamin K	Infectious hepatitis
Acetylsalicylic acid	Infectious mononucleosis
Nitrofurantoin	Bacterial pneumonias and septicemias
Furazoladone	(e.g. typhoid)
Diabetic acidosis	Uremia

monophosphate shunt. However, erythrocytes from G6PD-deficient individuals are incapable of producing sufficient NADPH by the hexose monophosphate shunt because they lack G6PD. This leads to oxidation of NADPH, glutathione, hemoglobin and other cellular components. Hemolysis is believed to be the ultimate result of oxidation of constituents of the cell membrane (20, 38, 39).

The presence of chemicals that can act as reducing agents or free radical scavengers appear to compensate for G6PD deficiency. *In vitro* supplementation with NADPH or ascorbate (41) was found to increase the GSH levels in G6PD-deficient erythrocytes. Furthermore, administration of vitamin E to G6PD-deficient individuals appears to lengthen the half-life of their erythrocytes and decrease their susceptibility to drug-induced hemolysis (42).

Notes

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Part 2. Final Report of the Investigator

Estimation of Risk of Glucose 6-Phosphate Dehydrogenase Deficient Red Cells to Ozone and Nitrogen Dioxide

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INTRODUCTION

The African variant of glucose 6-phosphate dehydrogenase deficiency (G6PD, A^- variant) is a sex-linked inheritable enzyme deficiency which affects approximately 14% of black males and 2% of black females in the United States. Dr. E.J. Calabrese et al. (1), working with extrapolations of data from published literature, has hypothesized that inhaled ozone might significantly decrease glutathione levels in G6PD-deficient erythrocytes (2), and therefore, that affected individuals might be at risk for significant hematologic effects due to inhalation of ambient levels of ozone or other oxidant species. To date, no experimental work to verify this hypothesis has been conducted; our study was designed to provide experimental data which could be used to evaluate whether or not ambient levels of oxidant gases might in fact pose a risk for individuals with G6PD deficiency.

The importance of G6PD for the normal metabolism and integrity of red blood cells came to be recognized as the result of the investigation of the unique sensitivity of some individuals to the 8-aminoquinoline antimalarial compound, primaquine. It was found that the observed sensitivity was due to a deficiency of the red cell enzyme G6PD (3). When primaquine is metabolized it produces metabolites which create an oxidant stress for the red blood cell. In the red cell, reduced glutathione is the major intracellular thiol active in the defense against free radicals and other oxidizing species. Because of the critical role which glutathione has in protecting the red cell from oxidant stress, it is necessary for the red cell to be capable of rapidly regenerating glutathione whenever it has been oxidized. When this is possible, the erythrocyte can continuously absorb free radicals and other oxidant species derived from drugs or other sources without harmful consequences to the cell. Oxidized glutathione is converted back to its reduced form via the action of glutathione reductase in the presence of NADPH. G6PD catalyzes the first enzymatic step in the hexose monophosphate shunt in which NADP is reduced to NADPH. In the red cell, which is deprived of an aerobic oxidation pathway, this represents the only means of generating NADPH and therefore is crucial. Hence, when the red cells of individuals with G6PD A^- deficiency are stressed by oxidants, they may not be able to generate enough NADPH to maintain an adequate concentration of reduced glutathione and the cell may ultimately undergo hemolysis.

Calabrese has made extrapolations to estimate the expected effect of ozone on red cell glutathione in man. Calabrese and his co-workers based their extrapolations on the report by Buckley et al. (2) of a 14% decrease in red cell glutathione in volunteers experimentally exposed to 0.5 ppm ozone for 2.75 hours. In comparison, the occupational standard for ozone is 0.1 ppm time-weighted average with a recommended short-term emergency limit of 0.3 ppm, and the primary air quality standard is 0.12 ppm for one hour. The second basis for the extrapolation by Calabrese was the studies of Zinkham et al. (4) demonstrating a four-fold greater decrement in intracellular glutathione of G6PD-deficient red cells incubated *in vitro* with acetylphenylhydrazine as compared to normal individuals. Calabrese et al. then reasoned that, were a deficient individual subjected to the level of ozone used by Buckley et al., there would be a four-fold greater decrement in red cell glutathione, i.e., a 56% decrement, which could be clinically significant.

AIMS

Hypothesis To Be Tested

Exposure of G6PD-deficient erythrocytes to ambient levels of ozone or nitrogen dioxide will result in a significant decrease in intracellular glutathione levels and/or the formation of Heinz bodies.

Specific Aims

To estimate the maximum extent to which ambient levels of ozone or nitrogen dioxide will affect the hematologic status of individuals with glucose 6-phosphate dehydrogenase deficiency (A^- variant) by comparing the effect produced by known oxidant drugs *in vivo* and *in vitro* to the effects produced by ozone and nitrogen dioxide in these systems.

METHODS AND STUDY DESIGN

In order to obtain some estimate of the potential effect of ambient levels of ozone or nitrogen dioxide on G6PD-deficient human red blood cells *in vivo*, we attempted to compare the effects of known oxidant drugs, such as phenylhydrazine, in both *in vivo* and *in vitro* systems, to the effects produced by either ozone or nitrogen dioxide in these systems. The primary *in vitro* model used was that of exposure of human G6PD-deficient erythrocytes to phenylhydrazine, ozone or nitrogen dioxide.

For these *in vitro* studies, the oxidation of reduced glutathione or the formation of Heinz bodies, rather than hemolysis, were used as endpoints. *In vivo* hemolysis depends in part upon the reticuloendothelial system which, of course, is not modeled *in vitro*. Hemolysis is a very late manifestation of *in vivo* red cell damage of the type caused by oxidant stress of G6PD-deficient cells. In contradistinction, glutathione loss and Heinz body formation are direct results of oxidant stress in G6PD deficiency and part of the pathogenic mechanism leading to

hemolysis *in vivo*. Note that when we speak of *significant* loss of glutathione we are referring to decreases of 50% or more from control values. Glutathione levels in normal, as well as G6PD-deficient individuals, usually range from 50-60 mg/100 ml packed red blood cells (mg%). It is only when glutathione levels fall below 20 mg% that the red cells appear unable to cope with oxidant stress (1, 4).

The blood for the *in vitro* studies was obtained from black male students at Rutgers Medical School. Memos were sent to students in the black community informing them of our study. We asked for volunteers who would be willing to be screened for G6PD deficiency; a total of 17 individuals volunteered. Blood was obtained with informed consent by standard venipuncture. The red cells were screened using a kit from Sigma based on the methods of Kornberg and Horecker, Bishop and Lohr and Waller. This is a kinetic enzyme assay and the change in absorbance at 340 nm was read at 30°C on a Perkin Elmer Model 552 spectrophotometer. Hemoglobin concentrations were determined by the standard cyanmethemoglobin method. Activity was reported in International Units (IU) of G6PD/gram hemoglobin.

Of the 17 volunteers screened, we identified four as being G6PD-deficient. Their levels of red cell G6PD (Table 2.1) were approximately 10% of normal values and ranged from 0.4 to 0.9 IU/gm Hb. During the course of our study, each time one of these individuals donated blood, his G6PD level was checked before beginning the experimental procedure; this was to insure that he had not had a recent hemolytic crisis. The presence of a large number of young red cells resulting from the reticulocytic response to a hemolytic event would tend to normalize the overall red cell G6PD activity, because the reticulocytes of individuals with the A^- variant of G6PD deficiency have completely normal levels of G6PD activity. However, in all cases, when we refer to human G6PD-deficient blood, we are specifically referring to blood whose red cell G6PD activity was in the range of 0.4-0.9 IU/gm Hb. The red cell G6PD activity of the four normal subjects ranged from 9.9-12.7 IU/gm Hb.

For the *in vitro* studies, human venous blood from donors was drawn into heparinized vacutainers; exposure of either control or G6PD-deficient red cells *in vitro* to oxidant species was always done on freshly drawn blood. For all blood samples drawn, the red cells were sedimented, washed three times in saline and diluted to a 5% hematocrit, in order to reduce viscosity, in phosphate-buffered saline pH 7.4 containing 5mM glucose. Reaction mixtures were handled identically whether the experimental drug was a liquid or gas; 10 ml of red cells were exposed to oxidant drugs using the methods described below, while incubated in a shaking water bath for two hours at 37°C. The reaction vessels were carefully monitored during the exposure

period to guard against volume loss due to evaporation. At the end of the incubation period, reduced glutathione was measured for control as well as for oxidant-treated samples by the procedure of Beutler et al. (5) In this procedure, the red cells are lysed and red cell proteins are acid precipitated. Nonprotein sulfhydryls are then measured in the protein-free filtrate with the disulfide compound 5,-5'-dithiobis (2-nitrobenzoic acid). In the red blood cell, where virtually all of the nonprotein sulfhydryl is in the form of reduced glutathione, this assay provides a fairly rapid as well as accurate means of quantitating red cell glutathione.

Ozone was generated in oxygen in an electric arc discharge generator and diluted with filtered room air to the appropriate concentration. Ozone concentrations in ppm were monitored continuously with a Pollution Control Industry (PCI) ozone analyzer, which monitors ozone concentration using an ultra violet absorption technique. Ozone was administered to the red cell suspension as a fine dispersion from a perforated glass tip dipping in the reaction mixture in a glass bubbler with ground glass fittings. The outlet was led to a potassium iodide (KI) trap. Administered ozone, as reported here, is the amount of ozone delivered to the reaction vessel minus the ozone which reacted in the KI trap.

Cylinders containing nitrogen dioxide (NO_2, 500 ppm in air) were purchased from Union Carbide Specialty Gas Corporation. The tank gas was diluted with filtered room air and the resultant concentration in ppm was monitored by the Saltzman Method at the standard 20 minute intervals. Nitrogen dioxide was administered to the red cell suspension as a fine dispersion from a perforated glass tip dipping in the reaction mixture in a glass bubbler with ground glass fittings. The outlet led to a trap which contained Griess-Saltzman absorbing solution (sulfanilic acid + N - (1-naphthyl)-ethylenediamine dihydrochloride), which is specific for nitrogen dioxide. Administered nitrogen dioxide, as reported here, is the amount of nitrogen dioxide delivered to the reaction vessel minus the nitrogen dioxide which reacted in the trap.

The *in vivo* studies used strain C57 L/J nine-month-old male mice (Jackson Labs, Bar Harbor, Maine), the same strain of G6PD-deficient mice used previously by Calabrese et al. (6) The red blood cells of these mice have G6PD activity levels of approximately 40-50% of normal strains (C57 Bl 6/J). G6PD levels in the mice used in our study were determined by using the same kit from Sigma Chemicals that was used for the human blood G6PD assays. Blacks with G6PD deficiency of the A^- variant typically have G6PD levels that are 8-15% of normal. Therefore, although these mice are G6PD-deficient, they are not deficient to the same extent as the human subjects.

The experimental mice were exposed to either ozone or nitrogen dioxide in a 5.5 cu. ft. plexiglass chamber. Chamber concentrations of ozone were monitored continuously with the PCI ozone analyzer; chamber concentrations of nitrogen dioxide were monitored by the Saltzman method at 20 minute intervals. The control animals were exposed to unfiltered ambient air. We modeled our study after that of Buckley et al., in which it was reported that exposure of human subjects to 0.5 ppm ozone for 2.75 hours resulted in a 14% decrease in blood glutathione levels immediately after exposure (2). We therefore exposed our G6PD-deficient mice to ozone or nitrogen dioxide for three hours, at doses ranging from ambient concentrations up to concentrations approaching the LD_{50} for these animals.

Immediately after exposure, blood glutathione levels were determined for these animals using the procedure of Beutler et al. (5) This method calls for blood in quantities that require the sacrifice of these small animals. Sacrifice of the mice, in turn, had two consequences. First, it was not possible to duplicate exactly the experimental design that Buckley used in the human study, in which it was possible to draw sufficient quantities of pre- and post-exposure blood from a single subject. Second, pre-exposure glutathione levels, for purposes of comparison with experimental post-exposure values, had to be determined by sacrificing animals from the control, or unexposed, group.

RESULTS

As shown in Table 2.1, the G6PD levels in our normal human subjects ranged from 9.9-12.7 IU/gm Hb, whereas those of our G6PD-deficient subjects ranged from 0.42-0.92 IU/gm Hb. The whole blood glutathione levels in the normal subjects (N1-N4) ranged from 56-62 mg%, and from 53-58 mg% in our G6PD-deficient subjects (D1-D4). Both measurements were found to be extremely reproducible from bleeding to bleeding in all subjects.

The normal C57 Bl 6/J mice had G6PD levels averaging 10.4 IU/gm Hb, while the G6PD-deficient C57 L/J strain had levels of 5.3 IU/gm Hb, or approximately 50% of control. The glutathione levels of the normal mice and those of the G6PD-deficient mice were in the range of 86.3 mg%. As expected, the variation in glutathione levels among these inbred animals was very small.

As shown in Table 2.2, there was no statistically significant difference in the blood glutathione levels of G6PD-deficient mice exposed to ozone and non-exposed G6PD-deficient controls. Note that the lungs of animals exposed to 4.1 and 5.2 ppm ozone for three hours showed signs of pulmonary edema, evidenced by increased lung weight, yet blood glutathione levels appeared to be unaffected.

Table 2.1
Baseline Glucose 6-Phosphate Dehydrogenase and Glutathione Levels (a) in Normal and Deficient Subjects

		Human (b)	
		G6PD (IU/gm Hb)	Glutathione (mg%)
Normal subjects:	N1	9.9 ± .10 (14)	61 ± 0.9 (14)
	N2	11.9 ± .14 (7)	62 ± 1.4 (7)
	N3	10.8 ± .33 (6)	59 ± 1.0 (6)
	N4	12.7 ± .12 (8)	56 ± 1.3 (8)
G6PD-deficient subjects:	D1	.42 ± .01 (14)	58 ± 1.0 (14)
	D2	.52 ± .01 (7)	57 ± 1.0 (7)
	D3	.58 ± .02 (6)	53 ± 1.4 (6)
	D4	.92 ± .01 (8)	57 ± 1.0 (8)
		Mouse (c)	
Normal (strain C57 Bl 6/J)		10.4 ± 0.5 (15) (d)	86.3 ± 7.4 (15) (d)
G6PD-deficient (strain C57 L/J)		5.3 ± 0.3 (15) (d)	86.3 ± 4.1 (105) (e)

(a) Mean ± standard error.
(b) For humans, the number in parentheses refers to the number of independent unexposed or pre-experimental determinations made on the same subject at different bleedings.
(c) For mice, the number in parentheses refers to the number of mice sacrificed for individual determinations to be made.
(d) One or two mice from each batch were tested when the batch was received, to verify either normalcy or G6PD deficiency of the entire batch.
(e) One or two mice from each batch received were tested, as well as the non-exposed animals in Tables 2.2 and 2.3 following.

Similarly, when G6PD-deficient animals were exposed to nitrogen dioxide (Table 2.3) in concentrations ranging from near-ambient (1.1 ppm) levels up to levels approaching the estimated LD_{50} for these animals, there was also no statistically significant decrease in the blood glutathione levels immediately following the acute exposures. Yet, at exposures of 250 ppm nitrogen dioxide these animals showed definite signs of pulmonary edema, many of them having frothy fluid in their noses and mouths.

When G6PD-deficient animals were injected with phenylhydrazine, a direct-acting oxidant, at a dose of 40 mg/kg, and their blood glutathione levels measured two hours after administration, exposed

Table 2.2
Red Cell Glutathione Levels in G6PD-Deficient Mice Exposed to Ozone

Ozone (a) (ppm)	Glutathione (b) (mg%) Non-exposed (c)	Exposed (c)
0.32	85.1 ± 3.3	89.8 ± 17.2
0.54	88.8 ± 11.8	96.3 ± 12.7
0.79	88.8 ± 11.8	79.5 ± 14.3
1.00	86.1 ± 7.2	91.7 ± 8.8
2.80	88.8 ± 11.8	86.2 ± 4.8
3.00	91.0 ± 5.0	99.7 ± 8.8
4.10	81.9 ± 6.7	86.5 ± 8.4
5.20	85.9 ± 10.7	87.9 ± 9.7

(a) Average concentration during a 3-hour exposure.
(b) Mean ± standard deviation.
(c) Twelve animals were used for each pair of measurements reported here; six were in the non-exposed group and six were in the exposed group. Using the Beutler method, each individual measurement required that the animal be sacrificed.

mice showed glutathione levels almost 50% lower than vehicle-treated controls (Table 2.4). In addition, these exposed animals also showed a 14% drop in hematocrit.

The results of *in vitro* studies with human red blood cells are presented in Tables 2.5, 2.6, and 2.7. From Table 2.5, it can be seen that average glutathione levels in G6PD-deficient subjects declined to 60% of baseline (control) levels following a two-hour incubation with 0.1 mM phenylhydrazine. Following incubation with 1.0 mM phenylhydrazine, average glutathione levels in these subjects was 40% of baseline. In contrast, for normal subjects, average glutathione levels were 82% and 75% of baseline levels following incubation with 0.1 mM and 1.0 mM phenylhydrazine, respectively.

As shown in Table 2.6, a total delivered dose of 4.28 micromoles of ozone decreased glutathione levels in G6PD-deficient cells by an average of 16%, compared to only 4% in the normal cells. Although this represents a statistically significant difference, (p .025 using Student's T test) we expect that a decrease of this magnitude would probably have

Table 2.3
Red Cell Glutathione Levels in G6PD-Deficient Mice Exposed to Nitrogen Dioxide

Nitrogen dioxide (a) (ppm)	Glutathione (c) (mg%) Non-exposed (d)	Exposed (d)
1.1	98.0 ± 13.8	87.6 ± 7.2
3.0	85.6 ± 9.2	97.3 ± 9.1
5.0	77.9 ± 8.4	82.8 ± 12.5
10.0	91.1 ± 1.9	89.2 ± 6.4
50	90.9 ± 15.7	84.5 ± 8.7
100	80.7 ± 13.2	89.1 ± 10.2
250 (b)	81.8 ± 13.5	89.4 ± 11.2

(a) Average level during a 3-hour exposure.
(b) Average level during a 2-hour exposure.
(c) Mean ± standard deviation.
(d) Twelve animals were used for each pair of measurements reported here; six were in the non-exposed group and six were in the exposed group. Using the Beutler method, each individual measurement required that the animal be sacrificed.

no clinical significance *in vivo*. Note that this result was produced by bubbling the red cell suspension with 5 ppm ozone flowing at a rate of 80 ml/min for four hours, and that the suspension's 5% hematocrit provided more ozone per red cell than would a suspension with a higher hematocrit. We chose these specifications after performing a series of nine separate *in vitro* studies in which total ozone delivered ranged from 0.084 micromoles to 2.14, and in which the hematocrits varied from 20% down to 5%; however none of these combinations yielded an effect on intracellular glutathione levels. As a result, we used 5% red cell suspensions and higher levels of ozone for longer periods in all our subsequent *in vitro* studies with ozone.

The results of our *in vitro* experiments with nitrogen dioxide are given in Table 2.7. As with the ozone experiments, a series of range-finding studies was also carried out for nitrogen dioxide in order to determine the dose levels that affected intracellular glutathione levels. As is shown in Table 2.7, a total delivered dose of 85.6 micromoles of nitrogen dioxide decreased glutathione levels in G6PD-deficient red cells by an average of 14%; that dose was delivered

Table 2.4
Glutathione Levels in Red Cells of G6PD-Deficient Mice Treated With Phenylhydrazine (a) *in Vivo*

	Hematocrit (c)			Glutathione (d)	
	Initial	Final	Change (e)	(mg%)	Change (f)
Control (b) (vehicle only; 4 mice)	49	49	0%	86.3 ± 4.1	0%
Phenylhydrazine (5 mg/kg; 4 mice)	49	49	0%	84.5 ± 5.9	-2%
Phenylhydrazine (15 mg/kg; 4 mice)	49	46	-6%	70.3 ± 3.8	-19%
Phenylhydrazine (40 mg/kg; 4 mice)	51	44	-14%	44.2 ± 12.2	-49%

(a) Phenylhydrazine administered as saline solution intraperitoneally.
(b) Four animals were used as vehicle controls and four were in each group treated with different doses of phenylhydrazine.
(c) Hematocrits on tail-vein blood were determined for each animal before and after treatment.
(d) Mean ± standard deviation; animal sacrificed two hours after administration of either vehicle or phenylhydrazine, and glutathione level measured using Beutler method.
(e) Percent decrease from initial hematocrit to final hematocrit; using tail-vein blood, both initial and final measurements could be made on the same animal.
(f) Percent decrease from initial glutathione levels to final glutathione levels, calculated using the baseline levels presented in Table 2.1 as the initial levels; for glutathione measurement, pre- and post-exposure measurements cannot be made on the same animals.

by bubbling 100 ppm nitrogen dioxide at a flow rate of 80 ml/min for four hours. Again, the differences between the normal and G6PD-deficient subjects is statistically significant, but not, we believe, of clinical significance in either group. Also, although the decrease in glutathione levels in ozone and nitrogen dioxide exposed G6PD-deficient red cells is statistically significant compared to the decrease seen in control cells, it should be noted that it required extremely high levels of each oxidant to produce these effects. Such levels would most likely never occur *in vivo*.

DISCUSSION: EXPERIMENTAL HYPOTHESIS

It was the aim of this project to determine if ambient levels of ozone or nitrogen dioxide could pose a risk for significant hematologic effects to individuals having a deficiency (A^- variant) in red blood cell glucose 6-phosphate dehydrogenase. To do this, we exposed G6PD-deficient animals to varying concentrations of these pollutants and looked for decreased levels of blood glutathione following exposure. We chose to monitor blood glutathione levels because a study by Buckley et al. (2) reported a decrease in glutathione levels in normal individuals following ozone exposure.

Table 2.5
Effect of Phenylhydrazine on Glutathione Levels in Normal and G6PD-Deficient Human Red Cells *in Vitro*

	Glutathione			
	Normal subjects		G6PD-deficient subjects	
Subject pairs (b)	**Control level (mg%)**	**Post-exposure (d) (as % of control)**	**Control level (mg%)**	**Post-exposure (d) (as % of control)**
First (N2/D1) (c)				
Phenylhydrazine (a)				
0	68 mg%	100%	62 mg%	100%
.1 mM	—	80%	—	57%
1.0 mM	—	78%	—	36%
Second (N4/D4) (c)				
Phenylhydrazine (a)				
0	60 mg%	100%	58 mg%	100%
.1 mM	—	84%	—	63%
1.0 mM	—	72%	—	44%

(a) Freshly-drawn red cells were washed and diluted to a 5% hematocrit in phosphate-buffered saline containing 5 mM glucose; 10 ml aliquots were incubated with phenylhydrazine for two hours at 37°C; control (unexposed) rbc dilutions were also incubated.
(b) One normal and one G6PD-deficient subject comprised each experimental pair. Each subject served as his own control. Experimental bloods were run in duplicate and the values reported here reflect the average of these duplicate determinations.
(c) Subject numbers shown are those listed in Table 2.1; subject numbers prefixed by N are normal subjects, reported in the left columns, and subjects prefixed by D are G6PD-deficient subjects, reported in the right columns.
(d) Percent of control values were measured directly by optical density using the Beutler method.

But we were unable to detect decreases in blood glutathione levels in G6PD-deficient animals acutely exposed *in vivo* to ambient levels of ozone or nitrogen dioxide (Tables 2.2 and 2.3). Furthermore, no significant decreases could be found even when these animals were exposed to levels which approached the LD_{50} of these pollutants. Yet, at these high levels, the animals showed definite signs of pulmonary edema.

Results of our work with normal and deficient human red blood cells exposed *in vitro* to ozone and nitrogen dioxide (Tables 2.6 and 2.7) were consistent with the *in vivo* findings. In G6PD-deficient red cells, a total dose of 4.28 micromoles of ozone, delivered over a four-hour period, decreased glutathione levels by an average of 16%, while a similarly-administered dose of 85.6 micromoles of nitrogen dioxide

Table 2.6
Effect of Ozone (a) on Glutathione Levels in Normal and G6PD-Deficient Human Red Cells (b) *in Vitro*

	Glutathione			
	Normal subjects		G6PD-deficient subjects	
Subject pairs (b)	Control levels (mg%)	Post-exposure (e) (as % of control)	Control levels (mg%)	Post-exposure (e) (as % of control)
First (N2/D1) (d)	59	94%	62	80%
Second (N4/D4) (d)	65	96%	58	86%
Third (N1/D2) (d)	60	98%	56	85%
Means	61.3	96%	58.6	84%

(a) Ozone flowing at 80 ml/min, 5 ppm x 4 hr; delivery was at a rate of 0.0178 micromoles/ min. Total delivered dose was 4.28 micromoles.
(b) Freshly-drawn red cells were washed and diluted to a 5% hematocrit in phosphate-buffered saline containing 5 mM glucose, 10 ml volume.
(c) One normal and one G6PD-deficient subject comprised each experimental pair. Each subject served as his own control. Experimental bloods were run in duplicate and the values reported here reflect the average of these duplicate determinations.
(d) Subject numbers shown are those listed in Table 2.1; subject numbers prefixed by N are normal subjects, reported in the left columns, and subjects prefixed by D are G6PD-deficient subjects, reported in the right columns.
(e) Percent of control values were measured directly by optical density using the Beutler method.

produced a 14% decrease. We expect that decreases of this order of magnitude are not clinically significant; typically, glutathione levels must decrease by more than 50% of normal levels, to less than 20 mg%, before red cells appear unable to cope with oxidant stress (1, 4). And our range-finding studies yielded no measurable responses in glutathione levels at doses less than the above and with hematocrits greater than 5%.

Finally, our studies with the direct-acting oxidant phenylhydrazine confirmed that an oxidant stress to the red cells of G6PD-deficient mice (*in vivo*, Table 2.4) and humans (*in vitro*, Table 2.5) will result in a significant decrease in red cell glutathione levels. Yet an effect of comparable magnitude is not observed at even high concentrations of ozone or nitrogen dioxide. Thus, the levels of ozone or nitrogen dioxide used in our studies do not appear to be a source of oxidant stress to the red cell.

Before these results can be related to the experimental hypothesis, there are several essential considerations. First, it may be important to

Table 2.7
Effect of Nitrogen Dioxide (a) on Glutathione Levels in Normal and G6PD-Deficient Human Red Cells (b) *in Vitro*

	Glutathione			
	Normal subjects		G6PD-deficient subjects	
Subject pairs (b)	Control levels (mg%)	Post-exposure (e) (as % of control)	Control levels (mg%)	Post-exposure (e) (as % of control)
First (N2/D1) (d)	62	100%	57	85%
Second (N4/D4) (d)	59	96%	60	84%
Third (N1/D2) (d)	64	98%	58	89%
Means	61.6	98%	58.3	86%

(a) Nitrogen dioxide flowing at 80 ml/min, 100 ppm x 4 hours; delivery was at a rate of 0.356 micromoles/min. Total delivered dose was 85.6 micromoles.
(b) Freshly-drawn red cells were washed and diluted to a 5% hematocrit in phosphate-buffered saline containing 5 mM glucose, 10 ml volume.
(c) One normal and one G6PD-deficient subject comprised each experimental pair. Each subject served as his own control. Experimental bloods were run in duplicate and the values reported here reflect the average of these duplicate determinations.
(d) Subject numbers shown are those listed in Table 2.1; subject numbers prefixed by N are normal subjects, reported in the left columns; and subjects prefixed by D are G6PD-deficient subjects, reported in the right columns.
(e) Percent of control values were measured directly by optical density using the Beutler method.

review basic hematology, particularly as it relates to G6PD deficiency. Normal red blood cells survive 120 days in the circulatory system without a nucleus or any protein-making machinery. Accordingly, no new G6PD molecules can be formed during the period. As is observed to a variable extent with other red cell enzymes, there is a decrease in G6PD activity as the red cell ages, so that older populations of red cells have far less activity than do reticulocytes, the earliest form of red cells in the circulation (8).

A crucial point about the African variant of G6PD deficiency is that the reticulocytes of these individuals show completely normal levels of G6PD activity. Thus young red cells from blacks with G6PD deficiency should not be any more susceptible to oxidant stress than red cells from non-deficient individuals. However, G6PD activity decays more rapidly over time in G6PD-deficient erythrocytes than in normal ones, so that older red cells in G6PD-deficient individuals are more susceptible to the risk of oxidizing stress than comparably aged cells in normal individuals.

On the other hand, this marked difference in G6PD activity between younger and older red cells accounts for the finding that continuous exposure to an oxidant drug at levels that initially produce significant hemolysis leads to the establishment of a steady state in which a normal hematocrit is achieved. Although total red cell activity in the African variety of G6PD deficiency is only 10% of normal, the presence of the large number of young red cells that constitute the reticulocytic response to an oxidizing hemolytic drug will tend to normalize overall red cell G6PD activity. And in fact, experimental studies in humans with the A^- variant of G6PD deficiency have shown that ingestion of 30 mg daily of the oxidant drug, primaquine, results in an initial decrement in the hematocrit from 45% to 35%, which is then followed by reticulocytosis and normalization of the hematocrit despite continued ingestion of 30 mg primaquine daily (9). Nor is this finding particularly surprising, in view of the fact that normal human bone marrow is capable of producing up to six times as many red cells as normal in response to hemolysis; thus red cell survival could conceivably be reduced from 120 days to as little as 20 days while maintaining a normal hematocrit.

Second, there are certain important characteristics of the chemistry and biology of ozone toxicity that must be considered when assessing risk. For one, only a small percentage, if any, of a highly reactive gas such as ozone or nitrogen dioxide can be expected to reach the alveolus, penetrate the lung epithelium and enter intracellularly into circulating red blood cells. Although effects of ozone on circulating red cells have been observed in animals, these effects, at all but the highest concentrations of ozone, appear to be restricted to the cell membrane.

For example, early work from our laboratory has shown that ozone exposure leads to a decrease in red cell acetylcholine-sterase, a membrane enzyme located on the outside of the red cell, in mice exposed to 8 ppm ozone for four hours (7). Although red cells from exposed animals in this experiment showed decreased glutathione, the decrease was not statistically significant. Accordingly, it seems unlikely that an oxidizing species derived from ambient levels of ozone in the workplace or elsewhere could penetrate the red cell membrane and react with intracellular glutathione.

For another, the stoichiometry of the reaction would seem to preclude a significant decrease in total red cell glutathione. Assume every molecule of ozone inhaled directly penetrates the lung, enters the circulating red cell, and reacts with glutathione. In 2.75 hours of breathing 0.5 ppm ozone, a maximum of 20.3 micromoles could be inhaled for an average man, assuming a respiratory rate of 15 breaths per minute and a tidal volume of 0.5 liters per minute, resulting in an alveolar ventilation of 315 liters per hour. Assuming that the average

Table 2.8
Summary of Effects of Oxidants on Glutathione Levels in Normal and G6PD-Deficient Human Red Cells (a) *in Vitro*

Dose		Glutathione (percent decrease from control) (e)	
Oxidant	Total dose delivered (micromoles)	Normal subjects	G6PD-deficient subjects
Phenylhydrazine (b)	0.1	18%	40%
Ozone (c)	4.28	4%	16%
Nitrogen dioxide (d)	85.6	2%	14%

(a) Freshly-drawn red cells were washed and diluted to a 5% hematocrit in phosphate-buffered saline containing 5mM glucose, 10 ml volume.
(b) Incubated for two hours at 37°C; 10 ml aliquot of phenylhydrazine at 0.1 mM/liter.
(c) Ozone flowing at 80 ml/min, 5 ppm x 4 hours; delivery was at a rate of 0.0178 micromoles/min.
(d) Nitrogen dioxide flowing at 80 ml/min, 100 ppm x 4 hours; delivery was at a rate of 0.356 micromoles/min.
(e) Percent decreases are averages for each oxidant species computed from Tables 2.5, 2.6, and 2.7.

human with G6PD deficiency has a glutathione level of 50 mg%, as described by Beutler (10), and further assuming normal body size and the relatively low hematocrit of 40%, then total blood glutathione would be about 4 millimoles. Since one molecule of ozone could react with two molecules of glutathione, the total decrease in red cell glutathione under these conservative assumptions would be 55 micromoles, or 1.4% of total red cell glutathione during the 2.75 hour period. This decrease is far less than the 56% suggested by Calabrese, and is in a range that would probably not be of hematological significance.

The results of our *in vitro* experiments on human red cells with the direct-acting oxidant drug and with ambient pollutants are summarized in Table 2.8. We believe that available evidence indicates that oxidant drugs are more potent stressors than ambient ozone. We further believe that, even if ozone were a far more potent stressor than it appears to be, the stoichiometry of ozone toxicity would preclude the appearance of clinically significant effects. Finally, even though the daily ingestion of 30 mg of an oxidant drug such as primaquine produces a substantial acute oxidant stress (one that is orders of magnitude greater than inhalation or 0.1 ppm ozone for eight hours), its chronic effects are nullified by hematologic compensatory mechanisms (this phenomenon is further discussed below). Accordingly, we conclude that it is highly

unlikely that ambient levels of ozone pose a risk of chronic anemia to individuals with A^- variant of G6PD deficiency.

DISCUSSION: SPECIFIC EXPERIMENTAL AIMS

We believe that our results are adequate to establish that the experimental hypothesis — that exposure of G6PD-deficient erythrocytes to ambient levels of ozone or nitrogen dioxide will result in a significant decrease in intracellular glutathione levels — is not valid. However, we were unable to quantify and refine these findings to the extent we had hoped. First, our experiments were confined to acute exposures; we were unable to study the effects of chronic exposure. Second, we were unable to develop the primaquine data that would have allowed us to link other published data sets, and thus predict ozone levels that would be required to produce hemolysis.

Specifically, we had originally planned to use the oxidant drug, primaquine, for our *in vivo* animal studies. We wanted to use a level of the drug in animals comparable, on a mg/kg basis, to that used in humans by Alving (9). In addition, we were hoping to find a dose which, when administered to the animals, would produce a hematologic response in two to four hours, in keeping with the duration of our ozone and nitrogen dioxide exposures.

But, in the human studies done by Alving, G6PD-deficient individuals who received 35 mg primaquine base daily for two weeks did not exhibit clinical hemolysis until the second or third day of drug administration. Likewise, in our animals (both normal and deficient) we could not find an effect on red cell glutathione within 2-4 hours of administration of a single dose by gavage of primaquine, even when we used a dose approaching the LD_{50} of the drug. Perhaps sufficient time had not elapsed for metabolism to occur; it is the metabolites of primaquine, rather than the parent compound, that exert the oxidant stress.

Instead, we chose to use phenylhydrazine, known to be a direct-acting oxidant. After a number of range-finding studies, we determined that 40 mg/kg of phenylhydrazine administered intraperitoneally produced about a 50% decrease in red cell glutathione two hours after administration to G6PD-deficient mice. In our *in vivo* study with phenylhydrazine, G6PD-deficient animals were given a total dose of 40 mg/kg in a single bolus i.p. (Table 2.4).

In clinical studies by Kellermeyer et al. (11) on G6PD-deficient black males, 90 mg of phenylhydrazine was administered daily for a minimum of ten days. The total delivered dose to an average 70 kg man was therefore 900 mg or 12.8 mg/kg, approximately one-third the dose we administered to our G6PD-deficient mice; we used this higher dose in order to observe an effect in a shorter time.

The phenylhydrazine dose used in the human trials produced an acute hemolysis which, as has already been discussed, was self-limiting because it destroyed only the older, less G6PD- active and therefore more susceptible red cells; the young cells were resistant to hemolysis. The hematocrit, which over an initial period of a few days dropped 15-30%, returned to normal even with continued administration of the drug. In our mice, we observed a 14% drop in hematocrit within two hours of drug administration. From this comparison, it can be seen that both systems, mouse and human, respond in a similar fashion.

We would therefore expect that, if ozone or nitrogen dioxide exposure created an oxidant stress for the red cells in exposed animals, such a stress would also manifest itself by a drop in hematocrit and red cell glutathione levels. However, even when the G6PD-deficient mice are exposed to levels of ozone or nitrogen dioxide which are equal to or greater than 40 times the allowable ambient concentrations set by the EPA, we do not see any of the clinical manifestations which are seen following the administration of phenylhydrazine. In other words, the administration of phenylhydrazine (or primaquine, which is still used clinically), apparently produces an oxidant stress which exceeds any stress which could probably be caused by ambient levels of ozone or nitrogen dioxide; the *in vitro* data, too, support the notion that, on a mole-by-mole basis, phenylhydrazine appears to be more potent than either ozone or nitrogen dioxide.

Under the best of circumstances, it is difficult to correlate *in vitro* observations with *in vivo* expectations. We were also unable to develop data for primaquine *in vitro*, so this usual difficulty has been further compounded for us. However, it is still possible to develop some reasonable extrapolations based on the *in vitro* results we were able to obtain.

Thus, alveolar ventilation in an average human at rest is 315 liters/hour. Therefore, the intake of pollutant in one hour for a person breathing 0.5 ppm ozone in air would be 7 micromoles. We can attempt to calculate the ozone dosage to the blood, assuming that the inhaled ozone (or nitrogen dioxide) can pass the alveolar epithelium, the basement membrane, and the capillary wall. If we take the blood volume as 5 liters, the dosage for a person breathing 0.5 ppm ozone for one hour would be 7 micromoles/5 liters, or 1.4 micromoles/liter blood. In our *in vitro* studies, the delivery of 4.3 micromoles of ozone to a 10 ml aliquot of a 5% suspension of G6PD-deficient red cells resulted in a 16% decrease in red cell glutathione (Table 2.6). If the same dose were delivered to an adult whose blood volume is 5 liters and hematocrit is 40%, this would represent a 4,000-fold dilution of the effect; i.e., glutathione would be depleted by 0.004%, which would have no clinical significance. Even if only one one-hundredth of the ozone

which was delivered to the reaction vessel actually reached the red blood cells in our *in vitro* system (i.e., 0.04 micromoles), and was responsible for the 16% decrease in red cell glutathione, it can be estimated that it would require 4,000 times as much ozone (1600 micromoles) to achieve a similar decrease *in vivo*. This would require breathing ozone at levels that exceed allowable ambient standards by a factor of 100, assuming that all of the ozone inhaled reached the blood.

Recently, Miller et al. (12) have attempted to develop a mathematical model which can be used to estimate the amount of ozone reaching the lower respiratory tract of man. In addition, they have attempted to estimate the amount of ozone which can reach the blood compartment. According to their model, the amount of inhaled ozone which reaches the blood ranges from 0.01% to 2.0% of the total amount inhaled, depending on the thickness of the pulmonary membrane. As stated previously, an individual breathing 0.5 ppm ozone for one hour would take in a total of 7 micromoles of ozone. If we use the Miller model and assume that 2% (i.e., the maximum dose) of ozone will reach the blood, the total amount reaching the blood compartment will be 0.14 micromoles. It required approximately 30 times as much ozone (4.2 micromoles) to cause a decrease in red cell glutathione in our *in vitro* system, which consisted of a 10 ml volume of 5% G6PD-deficient red cell suspension. If Miller is correct in estimating that, under normal circumstances, the maximum amount of ozone reaching the blood will be 2% of the total inhaled, then 210 micromoles of ozone would have to be inhaled in order for 4.2 micromoles to reach the blood. Assuming an average ventilation of 315 liters/hour, it would require acute exposures to levels of ozone which far exceed the current ambient standard in order to inhale 210 micromoles during the course of an eight-hour work day. Exposure to lower levels of ozone for extended periods — that is, chronic exposure to low levels — might also result in the inhalation of a total of 210 micromoles. However, the detoxification pathways that might be overwhelmed during acute exposure are less likely to be overwhelmed during chronic low-level exposure. In addition, the nature of the toxic products formed as the result of exposure may well vary with the dose delivered at any given instant.

CONCLUSIONS

In summary, we believe that neither extrapolations from available data, nor analysis of the basic hematology and toxicology support the hypothesis that individuals with G6PD deficiency, A^- variant, are at risk from levels of oxidant gases likely to be present in the workplace or general environment. We further recognize that both extrapolations from our data, as well as the model systems used in this study, provide only indirect evidence for these conclusions. However, we believe that our deductions are the strongest ones possible in the absence of controlled human exposure.

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Glossary

Acute hemolytic anemia	A condition in which, due to rupture of red blood cells, there is a rapid decline in the number of red cells in the blood.
Erythrocytes	Red blood cells.
Glucose 6-phosphate dehydrogenase	An enzyme that catalyzes the second reaction of the hexose monosposphate shunt. In this reaction, glucose 6-phosphate is converted to 6-phosphogluconolactose, with the concomitant reduction of NADP to NADPH.
Glutathione	An intracellular tripeptide which is very important in cellular metabolism and detoxification. Levels of glutathione are expressed as milligrams per 100 ml of red blood cells (mg%).
Heinz body	Granules in red blood cells, formed due to damage to hemoglobin.
Hematocrit	The volume of erythrocytes packed by centrifugation from a given volume of blood; expressed as the percentage of total blood volume which consists of erythrocytes.
Hemolysis	Rupture of red blood cells, usually leading to a decline in the hematocrit.
Heterozygote	An individual who has inherited two different genes (one from each parent) at the same locus on the chromosome.
Hexose monophosphate shunt	Also called the phosphogluconate pathway or the pentose phosphate pathway. A pathway utilized for degradation of glucose in the extramitochondrial cytoplasm, and for generation of reducing power in the form of NADPH.
Homozygote	An individual who has inherited identical genes from both the parents at a given locus on the chromosome.
In vitro	In a test-tube. Outside a living organism, in the laboratory.
In vivo	In a living organism.
Intraperitoneal administration	Injection of a substance into the peritoneal cavity, which is lined by the peritoneal membrane and contains all the abdominal organs except the kidneys.
NADP and NADPH	Nicotinamide adenine dinucleotide phosphate, oxidized (NADP) and reduced (NADPH) forms. Constituent of enzymes important in cellular respiration.

Osmotic fragility	Refers to sensitivity (fragility) of the cell membrane that causes cells rupture readily from changes in the concentration of dissolved substances in the medium surrounding them.
Pathogenesis	The development of a diseased state.
Stoichiometric	The conversion of reactants into products by an exact relationship.
X-linked	Refers to a gene located on the X-chromosomes. Human females have two X-chromosomes. Human males have only one X-chromosome.

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